STANDARD OPERATING PROCEDURE

Title: Immunofluorescence (IF) Microscopy using EVOS™ M5000 Imaging System

SOP#: M-137

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1. PURPOSE

This procedure will describe how to prepare antibodies/protein targets for immunofluorescence (IF). The procedure utilizes fluorescent-labeled antibodies to detect specific target proteins at the subcellular level, followed by imaging using the EVOS™ M5000 Imaging System to provide information such as protein expression and localization.

2. SCOPE

This procedure applies to all samples that are processed for immunofluorescence analysis using EVOS™ M5000 Imaging System. This procedure incorporates the Human Protein Atlas (HPA) IF staining protocol for adherent and suspension cells using a 96-well microplate format but uses the EVOS™ M5000 Imaging System. The fixation and permeabilization of cells, primary and secondary antibody incubations 4′,6-diamidino-2-phenylindole (DAPI) fluorescent nuclear staining and mounting procedures will be described in this SOP.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with cell culture, paraformaldehyde (PFA)/lab safety procedures and the use of the EVOS™ M5000 imaging system. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation. It is the responsibility of the analyst to follow the procedure and document any deviations and all observations in a laboratory notebook.

4. EQUIPMENT

- Invitrogen™ EVOS™ M5000 Imaging System; Thermo Fisher Scientific
- Standard Laboratory Cell Culture CO_2 Incubator
- Biosafety Cabinet/Hood Class II
- Laboratory Chemical Fume Hood
- Centrifuge with swing rotor for microplates, Eppendorf, Model # 5810 R
- Vortex
- pH meter
- Stirring Hot plate

5. MATERIALS

Use the following recommended materials, or equivalent:

- Greiner Sensoplate™ glass bottom multi-well plates; Millipore Sigma, Cat. # M4187-16EA
- Shi-fix™ coated 96-well microplate; Everest Biotech, Cat. # SB-Shifix96
- 250 ml beaker and magnetic stir bars
- Concentrated NaOH
- Concentrated HCl
- Deionized (DI) Water (MilliQ or better)
- 10X Phosphate Buffered Saline (PBS); Fisher Scientific, Cat. #BP399-1 (diluted to 1X with DI water)
- Superfibronectin; Millipore Sigma, Cat. # S5171-.5MG
- Heat Inactivated Fetal Bovine Serum (FBS), Life Technologies, Cat. # 16140071
- 16% Paraformaldehyde (PFA); VWR, Cat. # BT140770-10X10
- 4% PFA/1X PBS **Prepare as follows**:

  Always use lab coat, eye protection and gloves and perform work in a fume hood when preparing and using 4% PFA/1X PBS.

  1. Pre-heat 75 ml 10% FBS diluted with1X PBS in a 250 ml beaker to 60°C on a hot plate with magnetic stirrer.
  2. Add 25 ml 16% PFA to the warm FBS/PBS (Step 1) and continue stirring for 20 min at 60°C.
  3. Slowly add concentrated NaOH to the solution from Step 2 until the solution reaches pH ~11.
  4. Allow the solution to cool down to room temperature (~ 20°C).

5. Adjust pH to 7.2-7.3 using first concentrated HCl, and then diluted HCl for fine adjustment.
6. Aliquot solution and store at -20ºC until use. Thaw at room temperature right before use.

- Triton X-100 Solution; Millipore Sigma, Cat. # 93443-100ML
- DAPI; VWR, Cat. # 10180-614. **Prepare DAPI solution as follows:**
  1. Dissolve 10 mg of DAPI powder in 2 ml MilliQ water to a 5 mg/ml (14.3 mM) concentrated stock solution. Store at -20°C until needed.
  2. When ready to use thaw 5 mg/ml DAPI stock solution at room temperature, then combine 80 µl of 5 mg/ml DAPI stock solution with 920 µl MilliQ water to a pre-diluted 400 µg/ml DAPI solution. Aliquot and store extra vials at -20°C until needed.
  3. When ready to use thaw pre-diluted 400 µg/ml DAPI solution at room temperature to prepare 0.2 µg/ml DAPI with 1X PBS (0.2 µg/ml DAPI/1X PBS) for experiment. Discard any unused 0.2 µg/ml DAPI/1X PBS.
- Aluminum PCR Plate Sealers, Greiner Bio-One; VWR, Cat. # 82050-998
- Pipettes and tips
- Micro centrifuge and tubes
- 50 ml 0.2 µm filter units; Thermo Fisher Scientific, Cat. # 564-0020
- Glycerol; Thermo Fisher Scientific, Cat. # 15514011
- Glycerol/10X PBS. **Prepare as follows:**
  1. Add 5 ml 10X PBS to 45 ml of glycerol. Mix by inverting.
  2. Autoclave or filter sterilize.
  3. Store at Room Temperature

6. REAGENTS

6.1. For Rabbit or Mouse Monoclonal Antibody Evaluation

- Cells corresponding to the antibody or protein of interest such as cell lines (suspension or adherent) or equivalent. Purified monoclonal antibody corresponding to the protein of interest
- Mouse or rabbit anti-alpha tubulin marker; Abcam, Cat. # Ab7291 or Ab176560, respectively.
- Goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 555 secondary antibodies; Thermo Fisher Scientific, Cat. # A11034 and A21424, respectively or other species as appropriate conjugated Alexa Fluor secondary antibodies.

7. PROCEDURE

7.1. Guidelines

- All volumes in the protocol refer to volume per well in a 96-well microplate. Unspecified incubation temperatures are at room temperature. Immunostaining can be performed manually or using a liquid handling robot.

7.2. Preparation of Primary Antibodies

- Before immunostaining, dilute primary antibodies to 2 ug/ml in Blocking Buffer (4% FBS/1X PBS) together with 1ug/ml anti-alpha tubulin marker in one tube.

7.3. Preparation of Secondary Antibodies

- Before immunostaining, dilute secondary antibodies to 2.5 ug/ml in Blocking Buffer (4% FBS/1X PBS) and protect from light**.**

7.4. Cell Culture

- Grow adherent cells overnight in 80 µl cell culture media (according to cell provider's instructions) on fibronectin (12.5 µg/ml) coated 96-well glass bottom microplate (precoated according to manufacturer instructions). Note, optimal seeding concentration/confluency for IF staining varies between cell lines and test samples may be required to define optimal conditions for desired cell line/target. For suspension cells (10 million cells/mL in 1X PBS) use the Shi-fix™ coated 96-well microplate at a recommended concentration between 200,000 -500,000 cells/well. Follow Shi-fix™ manufacturer's instructions and proceed to fixation. Note, optimal cell count for IF staining varies between cell lines and test samples may be required to define optimal conditions for desired cell line/target.

7.5. Fixation

- Remove cell culture media (aspirate ~ 80 µl from all wells).
- Wash cells once gently with 40 µl 1X PBS.
- Fix cells by incubating with 40 µl of 4% PFA/1X PBS for 15 minutes.

7.6. Permeabilization

- Remove the 4% PFA/1X PBS and permeabilize the cells by incubating with 40 µl 0.1% Triton X-100/1X PBS for 5 minutes and repeat 3X.

- Remove Triton X-100/1X PBS and gently wash cells once with 40 µl 1X PBS.

7.7. Primary Antibody incubation

- Add 40 µl Blocking Buffer (4% FBS/1X PBS) with diluted primary antibodies and anti- alpha tubulin marker (Step 7.2).
- Incubate overnight at 4°C or for two hours at room temperature.

7.8. Secondary Antibody incubation

- Remove Blocking Buffer containing primary antibodies and gently wash cells with 40 µl 1X PBS for 10 minutes repeat 4X.
- Add 40 µl Blocking Buffer containing diluted secondary antibodies (Step 7.3)
- Incubate at room temperature for 90 minutes in the dark.

7.9. **DAPI Nuclear Staining and Mounting**

- Remove Blocking Buffer containing secondary antibody and incubate with 40 µl 0.2 ug/ml DAPI/1X PBS for 10 minutes.
- Gently wash the cells with 40 µl 1X PBS for 10 minutes repeat 4X.
- Add glycerol/10X PBS to cover the cells (~200 ul) and seal the plate with an adhesive aluminum PCR plate seal.
- Image the cells immediately or store plates at 4°C for no more than two weeks before imaging for best results.

8. REFERENCED DOCUMENTS

8.1 HPA Standard Immunostaining Protocol, https://www.protocols.io/view/hpa-cell-atlas-standard-immunostaining-protocol-x2dfqa6

8.2 Shi-fix™ coated 96-well microplate protocol, Everest Biotech Product Datasheet